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REMARKS

Claims 1, 2, 9-14 and 21-36 are pending. Claims 1, 2, and 9-20 were withdrawn from consideration. As suggested in the interview, Claim 21 has been limited to the specific diseases disclosed in Claim 22. Claim 22 has been further limited to dementia as described by original claim 4 and in the specification on page 6, lines 16-*et seq*. Accordingly, the Applicants do not believe that any new matter has been added.

The Applicants thank Examiner Jones for the courteous and helpful discussion of April 6, 2004. The Examiner indicated that he would likely withdraw the prior art rejection over Wang et al. because the compound of Wang, FK906 is excluded from the present claims. The enablement rejection was reviewed and the concern was that Claim 21 could encompass brain disorders not treatable with N-type calcium channel potentiation. To address the enablement rejection, it was suggested that the Applicants incorporate the limitations of Claim 22 into independent Claim 21 and, if possible, provide a document showing an association between particular brain disorders and N-type calcium channel potentitation. The Applicants have now made these amendments and provide herewith Catterall, Cell Calcium 2/6:307 (1998), which shows that calcium channels play a key role in neurological function. Favorable consideration is now respectfully requested.

REJECTION – 35 U.S.C. § 112, FIRST PARAGRAPH

Claims 21-36 were rejected under 35 U.S.C. § 112, first paragraph, as lacking adequate enablement for treatment of brain disorders in general, or for prevention of brain disorders in general. The Applicants thank Examiner for acknowledging that the claims are enabling for potentiating N-type calcium channel activity. As discussed in the interview, the Applicants have now limited the claims to specific diseases described in original Claim 4 and on page 6 of the specification.

The invention, as described in Claim 21, is directed to the discovery that compounds which exert an effect of specifically potentiating an N-type Ca^{2+} channel activity may be used to treat certain brain disorders. Treatment of brain disorders by potentiation of N-type calcium channel activity is described, for instance, on page 1, lines 5-11, page 3, lines 13-16, and page 4, lines 4-15 and page 8, lines 25-26. Catterall, Cell Calcium 2/6:307 (1998), also discloses that calcium channels play a key role in neurological function. Therefore, potentiation of these functions would be expected to remedy functional deficits associated with particular brain diseases. Moreover, based on the Catterall, the disclosed N-type calcium channel potentiating activity of the claimed compounds, and the recommended dosage ranges and routes of administration (see page 10), one with skill in the medical or physiological arts would be able to practice the invention without undue experimentation. Accordingly, the Applicants respectfully submit that this rejection would not apply to the present claims.

REJECTION – 35 U.S.C. § 103

Claims 21-36 were rejected under 35 U.S.C. § 103(a) as being unpatentable over Wang et al, Society for Neuroscience Abstracts, Vol. 26, Abstract No. 433. Wang discloses FK-506. However, Claims 21-36 **exclude** compounds of formula (I), such as FK960 of Wang et al. Accordingly, the Applicants respectfully request that this rejection be withdrawn.

CONCLUSION

In view of the above amendments and remarks, the Applicants respectfully submit that this application is now in condition for allowance. Early notification to that effect is earnestly solicited.

Respectfully submitted,

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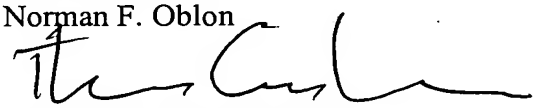

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Structure and function of neuronal Ca^{2+} channels and their role in neurotransmitter release

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Summary Electrophysiological studies of neurons reveal different Ca^{2+} currents designated L-, N-, P-, Q-, R-, and T-type. High-voltage-activated neuronal Ca^{2+} channels are complexes of a pore-forming α1 subunit of about 190–250 kDa, a transmembrane, disulfide-linked complex of α2 and δ subunits, and an intracellular β subunit, similar to the α1, α2δ, and β subunits previously described for skeletal muscle Ca^{2+} channels. The primary structures of these subunits have all been determined by homology cDNA cloning using the corresponding subunits of skeletal muscle Ca^{2+} channels as probes. In most neurons, L-type channels contain α_{1C} or α_{1D} subunits, N-type contain α_{1B} subunits, P- and Q-types contain alternatively spliced forms of α_{1A} subunits, R-type contain α_{1E} subunits, and T-type contain α_{1G} or α_{1H} subunits. Association with different β subunits also influences Ca^{2+} channel gating substantially, yielding a remarkable diversity of functionally distinct molecular species of Ca^{2+} channels in neurons.

L-type Ca^{2+} channels are primarily localized in cell bodies and proximal dendrites in neurons, and are present in clusters in the subsynaptic membrane of some glutamatergic synapses. They have a specific role in excitation–transcription coupling in neurons, but are not required for fast synaptic transmission. N-type and P/Q-type Ca^{2+} channels are localized in high density in presynaptic nerve terminals and are crucial elements in neuronal excitation-secretion coupling. In addition to mediating Ca^{2+} entry to initiate transmitter release, they interact directly with proteins of the synaptic vesicle docking/fusion machinery through a synaptic protein interaction (synprint) site in the intracellular loop connecting domains II and III of their α1 subunits. These interactions are calcium-dependent, channel-specific, and regulated by protein phosphorylation. These studies suggest that presynaptic Ca^{2+} channels not only provide the Ca^{2+} signal required by the exocytotic mechinery, but also contain structural elements that target docked synaptic vesicles to the source of Ca^{2+} influx and may be integral to vesicle docking, priming, and fusion processes.

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INTRODUCTION

Ca^{2+} channels in many different cell types activate upon membrane depolarization and mediate Ca^{2+} influx in response to action potentials and subthreshold depolarizing signals. Ca^{2+} entering the cell through voltage-gated Ca^{2+} channels serves as the second messenger of electrical signaling, initiating intracellular events such as contraction, secretion, synaptic transmission, and gene expression. Multiple types of Ca^{2+} currents have been identified in neurons by determination of their physiological and pharmacological properties. The relationship between the Ca^{2+} current types defined by these methods and cloned Ca^{2+} channel subunits characterized by expression *in vitro* is coming into clear focus, and the distinct subcellular localizations and functional roles of these different Ca^{2+} channel types are becoming well-defined. In this article, I review experiments which draw connections between Ca^{2+} current types defined physiologically and pharmacologically in neurons and the Ca^{2+} channel proteins defined by biochemical and molecular techniques, and I focus on emerging new insights into the role of Ca^{2+} channels in synaptic transmission in neurons.

Ca^{2+} CURRENT TYPES DEFINED BY PHYSIOLOGICAL AND PHARMACOLOGICAL PROPERTIES

Since the first recordings of Ca^{2+} currents in cardiac myocytes [1,2], it has become apparent that there are multiple types of Ca^{2+} currents as defined by physiological and pharmacological criteria [3–6] (Table 1). In cardiac, smooth, and skeletal muscle, the major Ca^{2+} currents are distinguished by high voltage of activation, large single channel conductance, slow voltage-dependent inactivation, marked regulation by cAMP-dependent protein phosphorylation pathways, and specific inhibition by Ca^{2+} antagonist drugs including dihydropyridines, phenylalkylamines, and benzothiazepines [7]. These Ca^{2+} currents have been designated L-type, as they are longlasting when Ba^{2+} is the current carrier [8]. L-type Ca^{2+} currents are also recorded in endocrine cells where they initiate release of hormones [9] and in neurons where they are important in regulation of gene expression and in integration of synaptic inputs[5].

Voltage clamp studies of Ca^{2+} currents in starfish eggs [10] and recordings of Ca^{2+} action potentials in cerebellar Purkinje neurons [11] first revealed Ca^{2+} currents with different properties from L-type, and these were subsequently characterized in detail in voltage-clamped dorsal root ganglion neurons [8,12–14]. In comparison to L-type, these Ca^{2+} currents activated at much more negative membrane potentials, inactivated rapidly, deactivated rapidly, had small single channel conductance, and were insensitive to Ca^{2+} antagonist drugs. They are designated low-voltage-activated Ca^{2+} currents for their negative voltage dependence [12] or T-type for their transient kinetics [8].

Whole-cell voltage clamp and single-channel recording from dissociated dorsal root ganglion neurons revealed an additional Ca^{2+} current, N type [8]. In these initial experiments, N-type Ca^{2+} currents were distinguished by their intermediate voltage dependence and rate of inactivation – more negative and faster than L type but more positive and slower than T-type [8,15,16]. They were insensitive to organic Ca^{2+} channel blockers but blocked by the cone snail peptide ω-conotoxin GVIA [3,17]. This pharmacological profile has been the primary method to distinguish N-type Ca^{2+} currents in recent work, because the voltage dependence and kinetics of N-type Ca^{2+} currents in different neurons vary considerably.

Analysis of the effects of other peptide toxins revealed three additional Ca^{2+} current types. P-type Ca^{2+} currents, first recorded in Purkinje neurons [18], are distinguished by high sensitivity to the spider toxin ω-agatoxin IVA [19]. Q-type Ca^{2+} currents, first recorded in cerebellar granule neurons [20], are blocked by ω-agatoxin IVA with lower affinity. R-type Ca^{2+} currents in cerebellar granule neurons are resistant to all of the organic and peptide Ca^{2+} channel blockers [20] and may include multiple channel subtypes [21]. While L-type and T-type Ca^{2+} currents are recorded in a wide range of cell types, N-, P-, Q, and R-type Ca^{2+} currents are most prominent in neurons and are therefore strong candidates for neuron-specific functions.

MOLECULAR PROPERTIES OF L-TYPE Ca^{2+} CHANNELS FROM SKELETAL MUSCLE

Ca^{2+} channels were first solubilized and purified from the transverse tubule membranes of skeletal muscle [22,23]. The initial purification studies revealed α1, β, and γ subunits and showed that the α1 and β subunits are substrates for cAMP-dependent protein phosphorylation [23,24]. More detailed biochemical analyses revealed an additional α2δ subunit co-migrating with the α1 subunit [25–28]. Analysis of the biochemical properties, glycosylation, and hydrophobicity of these five subunits led to a model comprising a principal transmembrane α_1 subunit of 190 kDa in association with a disulfide-linked $\alpha_2\delta$ dimer of 170 kDa, an intracellular phosphorylated β subunit of 55 kDa, and a transmembrane γ subunit of 33 kDa (Fig. 1A, [25]).

Table 1 Subunit composition and function of Ca^{2+} channel types

Ca^{2+} Channel Type	Localization	α_1 Subunits	Specific Blocker	Neuronal Functions
L	Cell bodies Proximal dendrites	α_{1S} α_{1C} α_{1D} α_{1F}	DHP's	Regulation of transcription
N	Nerve terminals Dendrites	α_{1B}	ω-CTx-GVIA	Neurotransmitter release Dendritic Ca^{2+} transients
P/Q	Nerve terminals Dendrites	α_{1A}	ω-Agatoxin	Neurotransmitter release Dendritic Ca^{2+} transients
R	Cell bodies Dendrites	α_{1E}	None	
T	α_{1G}	None α_{1H}	Repetitive firing	

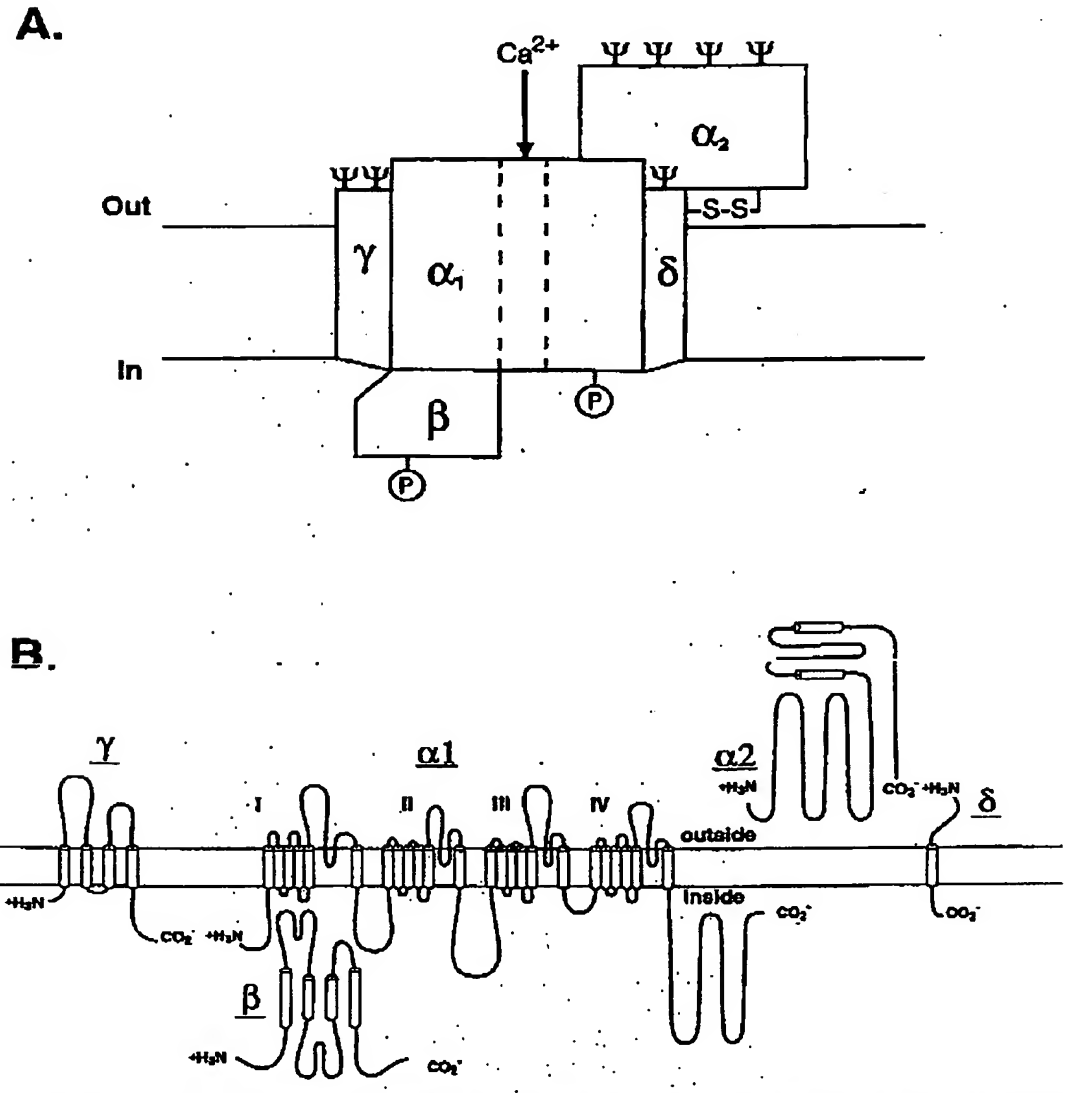

Fig. 1 Subunit structure of Ca^{2+} channels. (A) The subunit composition and structure of Ca^{2+} channels purified from skeletal muscle are illustrated. The model is updated from our original description of the subunit structure of skeletal muscle Ca^{2+} channels [25]. As described in the text, this model also fits biochemical and molecular biological results for neuronal Ca^{2+} channels. P, sites of phosphorylation by cAMP-dependent protein kinase. Ψ, sites of N-linked glycosylation. (B) Transmembrane folding models for the Ca^{2+} channel subunits. Predicted alpha helices are depicted as cylinders. The lengths of lines correlate approximately to the lengths of the polypeptide segments represented.

The primary structures of the five Ca^{2+} channel subunits were determined by combination of protein chemistry with cDNA cloning and sequencing. The α1 subunit is a protein of about 2000 amino acid residues with an amino acid sequence and predicted transmembrane structure like the previously characterized, pore-forming α subunit of sodium channels ([29], Fig. 1B). The amino acid sequence is organized in four repeated domains (I to IV) which each contains six transmembrane segments (S1 to S6) and a membrane-associated loop between

transmembrane segments S5 and S6. As expected from biochemical analysis [25], the intracellular β subunit has predicted alpha helices but no transmembrane segments ([30], Fig. 1B), while the γ subunit is a glycoprotein with four transmembrane segments ([31], Fig. 1B). The cloned α2 subunit has many glycosylation sites and several hydrophobic sequences [32], but biosynthesis studies indicate that it is an extracellular, extrinsic membrane protein, attached to the membrane through disulfide linkage to the δ subunit ([33], Fig. 1B). The δ subunit is encoded by the 3′ end of the coding sequence of the same gene as the α2 subunit, and the mature forms of these two subunits are produced by posttranslational proteolytic processing and disulfide linkage ([34,35], Fig. 1A & B).

Expression of the α1 subunit is sufficient to produce functional Ca^{2+} channels, but with low expression level and abnormal kinetics and voltage dependence of the Ca^{2+} current [36]. Co-expression of the α2δ subunit and especially the β subunit enhances the level of expression and confers more normal gating properties [37,38]. Intensive studies of the structure and function of related pore-forming subunits of sodium, calcium, and potassium channels have led to identification of the S4 segments of each homologous domain as the voltage sensors for activation and the S5 and S6 segments and the membrane-associated loop between them as the pore lining of the voltage-gated ion channels (reviewed in [39–41]). The sections below review the relationship between the subunit components of different neuronal Ca^{2+} channel subunits and the Ca^{2+} currents which they mediate.

SUBUNITS OF NEURONAL Ca^{2+} CHANNELS

Purification and immunoprecipitation studies of high-voltage-activated Ca^{2+} channels in neurons identified similar subunit components to those of skeletal muscle Ca^{2+} channels. Immunoprecipitation of L-type Ca^{2+} channels labeled by dihydropyridine Ca^{2+} antagonists revealed α1, α2δ, and β subunits but no γ subunit [42]. Purification and immunoprecipitation of N-type Ca^{2+} channels labeled by ω-conotoxin GVIA revealed α1, α2δ, and β subunits [43,44]. Similarly, purified P/Q-type Ca^{2+} channels also are composed of α1, α2δ, and β subunits [45–47]. However, recent experiments have unexpectedly revealed a novel γ subunit which is the target of the *stargazer* mutation in mice [48]. This γ-subunit-like protein can modulate the voltage dependence of expressed Ca^{2+} channels containing α_{1A} subunits, so it may be associated with P/Q-type Ca^{2+} channels in vivo [48]. If this new γ subunit is indeed associated with neuronal Ca^{2+} channels, their subunit composition would be identical to that of skeletal muscle Ca^{2+} channels defined in biochemical experiments [25] (Fig. 1).

As for skeletal muscle Ca^{2+} channels, co-expression of β subunits has a large effect on the level of expression and the voltage dependence and kinetics of gating of neuronal Ca^{2+} channels. In general, the level of expression is increased and the voltage dependence of activation and inactivation is shifted to more negative membrane potentials, and the rate of inactivation is increased. However, these effects are different for individual β subunit isoforms (reviewed in [49,50]). In contrast, co-expression of α2δ subunits [49,50] and γ subunits [48] has much smaller effects.

RELATIONSHIP BETWEEN NEURONAL Ca^{2+} CHANNEL TYPES DEFINED BY FUNCTION AND BY STRUCTURE

The different types of Ca^{2+} currents are primarily defined by different α1 subunits. The primary structures of eight distinct classes of neuronal Ca^{2+} channel α_1 subunits have been defined by homology screening and their function has been characterized by expression in mammalian cells or *Xenopus* oocytes (Table 1). These subunits have been designated classes A through H. L-type Ca^{2+} currents in neurons are mediated by Ca^{2+} channels containing α_{1C} and α_{1D} subunits, which have about 75% amino acid sequence identity with the α1 subunit of skeletal muscle L-type Ca^{2+} channels [51–53]. Recently, a third L-type α1 subunit (α_{1F} has been identified in retina as the target of mutations which cause stationary night blindness [54]. Expression of this α1 subunit appears to be restricted to retina, but expression in small subsets of other neurons is not excluded. The α1 subunit of skeletal muscle Ca^{2+} channels, α_{1S}, together with α_{1C}, α_{1D}, and α_{1F} comprise the first structural family of Ca^{2+} channel α1 subunits (Table 1).

The α_{1A}, α_{1B} and α_{1E} subunits form a distinct subfamily with less than 40% amino acid sequence identity with the L-type Ca^{2+} channel α1 subunits but greater than 70% amino acid sequence identity among themselves (Table 1). Cloned neuronal α_{1B} subunits form N-type Ca^{2+} channels with high affinity for ω-conotoxin GVIA [55,56]. Cloned α_{1A} subunits [57,58] form P- or Q-type Ca^{2+} channels which are inhibited by ω-agatoxin IVA [59,60]. Consistent with the idea that α_{1A} forms both P-type and Q-type channels, coexpression with appropriate β subunits can change the kinetics of channel gating to fit either P-type or Q-type characteristics [60], and immunocytochemical studies show that the α_{1A} subunit is localized in synapses where transmission is mediated by either P-type or Q-type channels [61]. The main difference in pharmacology of P-type and Q-type channels depends on alternative mRNA splicing. One alternatively spliced isoform with an insertion in the extracellular loop IVS3-S4 has relatively low affinity for ω-agatoxin IVA like

Q-type Ca^{2+} currents recorded in neurons, while a second isoform without that insertion has high affinity for ω-agatoxin IVA like P-type Ca^{2+} currents recorded in neurons (E. Bourinet et al., European Winter Conference on Brain Research, Abstract). Cloned α_{1E} subunits from R-type Ca^{2+} channels which are resistant to both organic Ca^{2+} antagonists and presently known peptide toxins [20,62,63]. They have relatively rapid voltage-dependent inactivation like R-type Ca^{2+} channels recorded in cerebellar granule neurons. Therefore, the α_{1E} subunit is the first example of an α1 subunit of high-voltage-activated Ca^{2+} channels which is resistant to currently available pharmacological agents. It is likely that other resistant (R-type) channels will be discovered, since physiological evidence indicates multiple single channel currents which are R-type [21]. Together, the α_{1A} through α_{1F} subunits can account for all of the high-voltage-activated Ca^{2+} currents that have been recorded and well characterized to date, although it is to be expected that more homologs may be found in specific types of neurons or other types of cells. However, none of these α1 subunits form Ca^{2+} channels with the characteristics of T-type.

A major gap in our understanding of neuronal Ca^{2+} channels was filled with the recent cloning and characterization of the α_{1G}, α_{1H}, and α_{1I} subunits, based on gene sequences discovered in the *C. elegans* and human genome projects [64]. These α1 subunits are only distantly related to the other known homologs, with less than 25% amino acid sequence identity. Expression of the α_{1C} subunit alone in a mammalian cell line generates Ca^{2+} currents with the negative voltage dependence of activation and inactivation, rapid inactivation, rapid deactivation, and pharmacology of T-type Ca^{2+} currents recorded in neurons and other cell types. These results reveal a surprising structural dichotomy between the T-type, low-voltage-activated Ca^{2+} channels and the high-voltage-activated calcum channels. Evidently, these two lineages of Ca^{2+} channels diverged very early in evolution of multi-cellular organisms.

The diversity of neuronal Ca^{2+} channel structure and function is substantially enhanced by multiple β subunits. Four β subunit genes have been identified, and each is subject to alternative splicing to yield additional isoforms (reviewed in [49,65]). In Ca^{2+} channel preparations isolated from brain, each Ca^{2+} channel α1 subunit that has been investigated is associated with multiple β subunits, although there is a different rank order in each case [66,67]. The different β subunit isoforms cause different shifts in the kinetics and voltage dependence of gating, so association with different β subunits can substantially alter the physiological function of an α1 subunit. In contrast, only a single gene encoding α2δ subunits has been described, and α2δ isoforms produced by alternative splicing have not been shown to have strong functional effects.

SUBCELLULAR LOCALIZATION DEFINES FUNCTION OF HIGH VOLTAGE-ACTIVATED Ca^{2+} CHANNELS IN CENTRAL NEURONS

The diversity of Ca^{2+} channels in neurons revealed by cDNA cloning and sequencing might suggest that different major neuron classes would express an individual Ca^{2+} channel subtype or perhaps a subset of Ca^{2+} channel subtypes. Quantitative differences in the level of expression of different Ca^{2+} channel types have been observed by electrophysiological analysis of dissociated neuronal cell bodies [19,68] and by Northern blotting and in situ hybridization analysis of mRNA expression levels. However, the dominant theme revealed by immunocytochemical studies of the distribution of Ca^{2+} channel subtypes in the different functional compartments of neurons is that most of the high voltage-activated Ca^{2+} channels are expressed in major neuronal cell types at significant levels but are localized in different regions of the cell to serve different functions. The theme that localization determines function is illustrated for the L-type, N-type, and P/Q-type Ca^{2+} channels in the sections below.

FUNCTIONAL ROLE OF L-TYPE Ca^{2+} CHANNELS IN NEURONS

Using monoclonal antibodies against purified skeletal muscle Ca^{2+} channels, cross-reacting L-type Ca^{2+} channels in neurons were found localized primarily in cell bodies and proximal dendrites of hippocampal pyramidal neurons and several other classes on neurons [42,69]. Consistent with this localization, L-type Ca^{2+} channels have a dominant role in Ca^{2+} influx into the cell bodies of CA3 neurons in organotypic hippocampal slices [70]. These L-type channels were not detected in high density in nerve terminals, arguing against an important role in neurotransmitter release.

Following cloning and functional analysis of the neuronal L-type Ca^{2+} channels, anti-peptide antibodies which specifically recognize the α_{1C} and α_{1D} subunits were developed and shown to be specific for L-type Ca^{2+} channels [55]. Immunocytochemical analysis of the distribution of these two α1 subunits revealed a distinct difference in subcellular localization [71]. Although both channel types were predominantly localized in cell bodies and proximal dendrites, α_{1D} subunits were smoothly distributed along the cell surface at the resolution of the light microscope while α_{1C} subunits were localized in approximately 1 μm clusters on cell bodies and dendrites, and clusters of α_{1C} extended far out the dendrites of

many neuron types, including CA3 pyramidal neurons [71] (Fig. 2). Clusters of α_{1C} could be observed in the postsynaptic membrane of asymmetric, glutamatergic synapses in hippocampal neurons, and activation of the NMDA subtype of glutamate receptors in hippocampal slices in vitro caused proteolytic processing of the COOH terminal domain of α_{1C} [72]. These results suggest local Ca^{2+} signaling events between Ca^{2+} entering through activated NMDA receptors and nearby L-type Ca^{2+} channels. Truncation of the COOH terminal domain of α_{1C} increases its Ca^{2+} conductance activity when expressed in *Xenopus* oocytes [73], so this local Ca^{2+} signal could enhance L-type Ca^{2+} channel activity in postsynaptic membranes after activation of NMDA receptors by synaptically released glutamate.

What neuronal functions might be regulated by Ca^{2+} influx into the cell body and dendrites through L-type Ca^{2+} channels? Several lines of evidence indicate that L-type Ca^{2+} channels have a crucial role in regulation of gene transcription. In cultured cortical neurons, activation of transcription of immediate early genes by repetitive electrical activity depends primarily on Ca^{2+} entry through L-type Ca^{2+} channels [74]. Activation of the cAMP- and calcium-dependent transcription factor CREB (cAMP response element binding protein) by cellular depolarization requires Ca^{2+} influx through L-type Ca^{2+} channels, and influx through L-type channels was quantitatively more effective in activation of CREB than comparable influx through NMDA receptors [75,76]. Long-lasting long-term potentiation in hippocampal neurons in cell culture and hippocampal slices requires activation of NMDA receptors and Ca^{2+} influx through L-type Ca^{2+} channels which activates CREB by phosphorylation by calcium–calmodulin kinase IV [77–79]. Ca^{2+} entry through L-type Ca^{2+} channels is more effective than Ca^{2+} entry through other pathways in this transcriptional activation, and calmodulin is proposed as a second messenger which moves from the cell surface to the nucleus to initiate activation of CREB [77,80,81]. Together, these results point to the L-type Ca^{2+} channel as a critical element in excitation-transcription coupling in neurons. These studies of regulation of gene transcription by synaptic input and activation of L-type Ca^{2+} channels are described in more detail in the article by Tsien in this issue.

Ca^{2+} CHANNELS AND SYNAPTIC TRANSMISSION IN NEURONS

Neurotransmitter release is initiated by influx of Ca^{2+} through voltage-dependent Ca^{2+} channels within 200 μs of the arrival of the action potential at the synaptic terminal [82], where clusters of presynaptic Ca^{2+} channels are thought to supply Ca^{2+} to initiate release [83–86]. Exocytosis of synaptic vesicles requires high Ca^{2+} concentration, with a threshold of 20–50 μM and half-maximal activation at 190 μM [87,88]. The brief rise in Ca^{2+} concentration to the level necessary for exocytosis likely occurs only in proximity to the Ca^{2+} channels [89,90], since intracellular Ca^{2+} concentration falls off steeply as a function of distance away from the Ca^{2+}

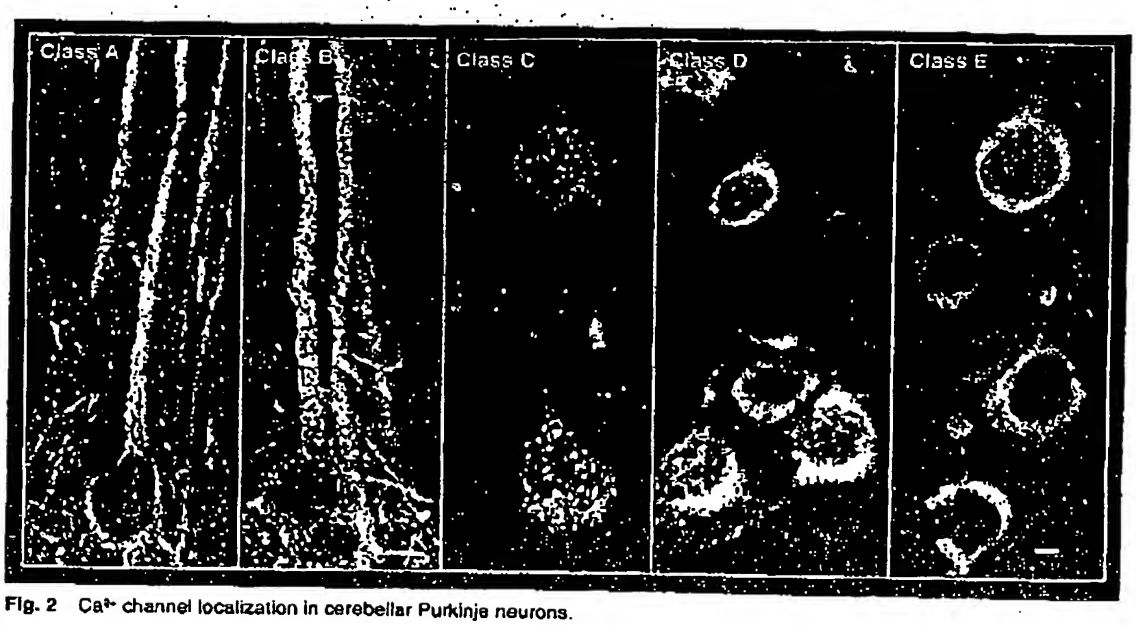

Fig. 2 Ca^{2+} channel localization in cerebellar Purkinje neurons.

channels. Thus, a precise organization of presynaptic Ca^{2+} channels and the exocytotic machinery is expected in nerve terminals. A more detailed presentation of biophysical studies of exocytosis is given in the article by Almers in this issue.

Major progress has been made towards understanding the molecular mechanisms that underline Ca^{2+}-dependent exocytosis by identifying proteins that are involved in the vesicle docking/fusion process at presynaptic nerve terminals and analyzing their interactions (reviewed in [91,92]. Vesicle docking and fusion involve the synaptic vesicle SNARE protein VAMP/synaptobrevin [93] and the plasmalemmal SNARE proteins syntaxin [94,95] and SNAP-25 [96] which form a stable core complex [97–100]. Recent evidence indicates that these SNARE proteins form coiled-coil hairpin structures that can cause vesicle hemifusion and mixing of lipid contents by themselves [101,102]. The intrinsic synaptic vesicle protein synaptotagmin (originally discovered as p65 [103]) binds Ca^{2+} and interacts with syntaxin in a Ca^{2+}-dependent manner [104–108]. It is thought to serve as a Ca^{2+} sensor for fast, Ca^{2+}-dependent neurotransmitter release [109–113]. Ca^{2+} entry through presynaptic Ca^{2+} channels is likely to trigger fast exocytosis by binding to synaptotagmin and other associated presynaptic proteins.

LOCALIZATION OF Ca^{2+} CHANNELS IN NERVE TERMINALS

In contrast to L-type Ca^{2+} channels, there is strong evidence for localization of N-type and P/Q-type Ca^{2+} channels in nerve terminals. At the frog neuromuscular junction, high affinity binding sites for ω-conotoxin GVIA, which labels N-type Ca^{2+} channels, are localized in the presynaptic nerve terminals in the position of active zones [85]. In mammalian neuromuscular junctions, high affinity binding sites for ω-conotoxin MVIIC, which labels primarily P/Q-type Ca^{2+} channels, are similarly localized [114]. The α_{1B} subunits of N-type Ca^{2+} channels and the α_{1A} subunits of P/Q-type Ca^{2+} channels [61,115] are localized at low density in dendrites and at high density in presynaptic nerve terminals of many central neurons (Fig. 2). The high density clusters of α_{1A} subunits and α_{1B} subunits in presynaptic nerve terminals are precisely colocalized with high density clusters of the SNARE protein syntaxin [61]. Thus, the α_{1A} and α_{1B} subunits are co-localized in nerve terminals with the SNARE proteins which carry out synaptic vesicle docking and exocytosis.

PHYSIOLOGICAL ROLE OF Ca^{2+} CHANNEL SUBTYPES IN SYNAPTIC TRANSMISSION

Experiments with specific pharmacological agents indicate that fast synaptic transmission as measured electrophysiologically requires N-type and/or P/Q-type Ca^{2+} channels in essentially all fast synapses that have been studied to date, except the specialized ribbon synapses of visual and auditory systems. In the first study of this kind, Kerr and Yoshikami [116] showed that ω-conotoxin GVIA potently inhibits transmission at the frog neuromuscular junction. Similarly, release of norepinephrine from sympathetic neurons is blocked by ω-conotoxin GVIA [117]. These results and much subsequent work indicate that N-type Ca^{2+} channels are the dominant source of Ca^{2+} influx at many peripheral synapses (reviewed in [118]). In contrast, at many central synapses synaptic transmission is blocked more effectively by agents which target P/Q-type Ca^{2+} channels [118]. ω-agatoxin IVA- and ω-conotoxin MVIIC-sensitive P/Q-type Ca^{2+} channels are more effective in mediating transmission than ω-conotoxin GVIA-sensitive N-type Ca^{2+} channels at synapses between dentate granule neurons and hippocampal CA3 neurons [119], CA3 and CA1 hippocampal pyramidal neurons [120–122], or cerebellar granule neurons and Purkinje neurons [123]. In contrast, interneurons in the hippocampus use either N-type Ca^{2+} channels or P/Q-type Ca^{2+} channels but not both [124]. Detailed analysis of the concentration dependence of inhibition of synaptic transmission by ω-agatoxin IVA has been used to provide evidence that Q-type rather than P-type Ca^{2+} channels mediate transmission at synapses between CA3 and CA1 neurons in the hippocampus [121]. These experiments are made difficult by the slow action of the toxins and the diffusion barriers and limited stability of the experimental preparations used, so the distinction between P-type and Q-type Ca^{2+} channels in mediating transmitter release at central synapses remains unsettled [125]. Nevertheless, it is clear that N-type Ca^{2+} channels containing α_{1B} subunits and P-type and/or Q-type Ca^{2+} channels containing α_{1A} subunits are dominant in mediating fast synaptic transmission at virtually all conventional synapses. What is the molecular basis for their specific role in fast synaptic transmission?

SYNAPTIC PROTEIN INTERACTION (SYNPRINT) SITE ON N-TYPE Ca^{2+} CHANNELS

Biochemical and immunochemical studies indicate a tight association of syntaxin and synaptotagmin with both N- and P/Q-type Ca^{2+} channels extracted from brain membranes [94,95,126–129], suggesting both types of Ca^{2+} channels interact with components of synaptic vesicle docking/fusion machinery. To identify the cytoplasmic loops of the N-type channels interacting with synaptic proteins, we constructed a series of hexahistidine-tagged (His)-fusion proteins containing each of the cytoplasmic segments of the α_{1B} subunit of N-type Ca^{2+} channels and glutathione S-transferase (GST)-tagged recombinant syntaxin 1A, SNAP-25, or VAMP. The

recombinant GST-fusion proteins coupled to glutathione-Sepharose beads were used as an affinity matrix to screen the His-tagged Ca^{2+} channel fusion proteins for specific binding. The *in vitro* binding studies showed that both syntaxin 1A and SNAP-25, but not VAMP, specifically interact with the cytoplasmic loop (L_{II-III}) between homologous domains II and III of the α_{1B} subunit of N-type Ca^{2+} channels through binding sites formed by residues 718–963 [130,131]. This site on N-type Ca^{2+} channels contains two adjacent binding regions which can each bind synaxin. We use the term 'synprint' to designate this *syn*aptic *p*rotein *int*eraction site on presynaptic Ca^{2+} channels (Fig. 3). The synprint peptide can specifically block co-immunoprecipitation of native N-type Ca^{2+} channels with syntaxin, indicating that this binding site is required for stable interaction of these two proteins [130]. This interaction takes place with the C-terminal one-third of syntaxin (amino acid residues 181–288), suggesting that neuronal Ca^{2+} channels bind to syntaxin 1A at a C-terminal site near the intracellular surface of the plasma membrane [130,132].

Ca^{2+} DEPENDENCE OF INTERACTIONS WITH THE SYNPRINT SITE ON N-TYPE Ca^{2+} CHANNELS

An important unresolved issue in understanding neurotransmitter release is the mechanism of its Ca^{2+} dependence. Exocytosis requires high Ca^{2+} concentration, with a threshold of 20–50 μM and half maximal activation at 190 μM [87,88]. Synaptotagmin may be the low-affinity Ca^{2+}-sensor since it binds to syntaxin and phospholipid in a Ca^{2+}-dependent manner in the range of 10–50 μM and 100–300 μM Ca^{2+}, respectively [104,133,134], and inhibition of synaptotagmin function in cultured cells or deletion of synaptotagmin genes in mice, fruit flies, and nematodes impairs synchronous synaptic transmission [108,110,112,135]. However, other Ca^{2+} responsive proteins may also be involved in the docking/fusion process as residual neurotransmission persists in these synaptotagmin-null mutants [110,112,135].

To find additional Ca^{2+}-responsive interactions among synaptic proteins and Ca^{2+} channels, we measured the binding of these recombinant proteins *in vitro*. We found that the interaction of the N-type synprint peptide with recombinant syntaxin, SNAP-25, or the synaptic core complex of syntaxin-SNAP-25-VAMP/synaptobrevin has a biphasic dependence on Ca^{2+} concentration, with maximal binding at approximately 20 μM free Ca^{2+} [131]. This Ca^{2+}-dependent interaction takes place in the same concentration range as the threshold for fast transmitter release. Thus, the direct interaction of presynaptic Ca^{2+} channels with the synaptic fusion core-complex is a Ca^{2+}-sensitive process and may play a key role in docking and/or fusion of synaptic vesicles. The steps of interaction and dissociation of the complex of N-type Ca^{2+} channels with SNARE proteins as a function of Ca^{2+} concentration are illustrated in Figure 4.

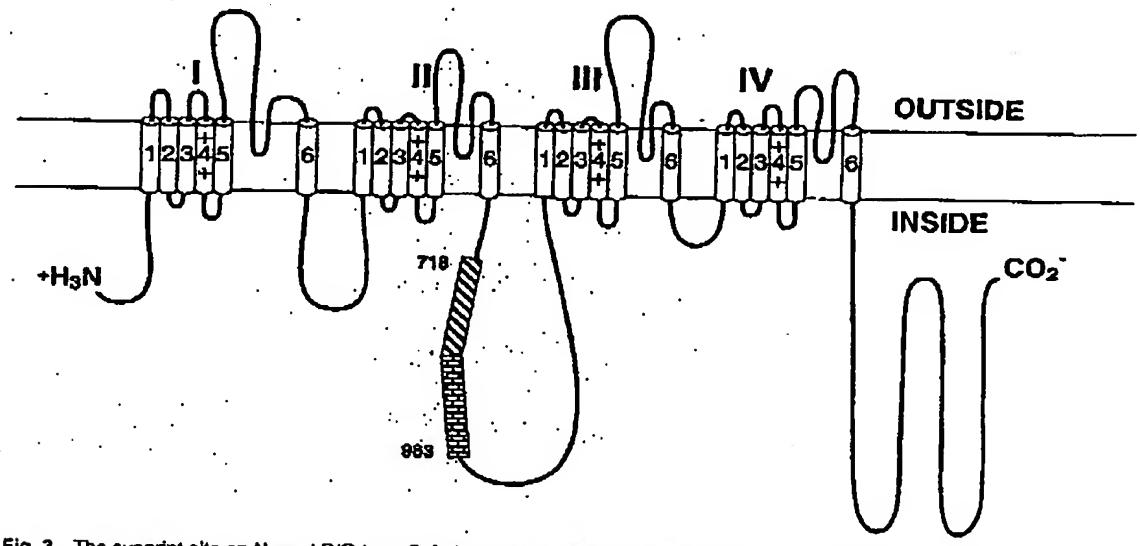

Fig. 3 The synprint site on N- and P/Q-type Ca^{2+} channels. Predicted topological structure of the α_1 subunits of class B N-type and class A P/Q-type Ca^{2+} channels with synprint site in the intracellular loop between homologous domains II and III (L_{II-III}) indicated by the rectangle boxes. Amino acid positions of N-, and P/Q-type synprint sites are defined in the regions between 718–963 and 722–1036, respectively.

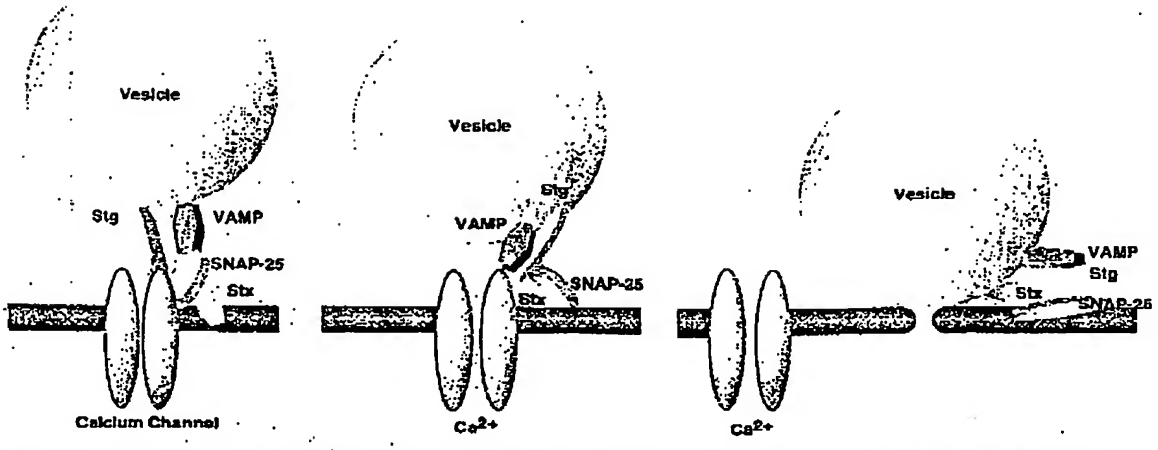

Fig. 4 The proposed model for the sequential Ca^{2+}-dependent interactions of the synprint site of N-type Ca^{2+} channels with multiple SNARE proteins during synaptic vesicle docking/fusion process. (A) The pre-docked vesicles form a low-affinity complex with the N-type Ca^{2+} channels through binding to syntaxin and SNAP-25 at resting [Ca^{2+}] level (<10 μM). (B) The initial Ca^{2+} influx (10–20 μM) greatly increases the affinity of this coupling, so the binding energy of Ca^{2+} to this complex may contribute to the energetic driving force for the early priming steps of the fusion process. (C) Finally, as the free Ca^{2+} reaches the threshold for release (>20 μM), the binding affinity of this coupling is reduced, and syntaxin and SNAP-25 dissociate from the channels. Higher levels of Ca^{2+} (above 30 μM) may be needed to enable displacement of syntaxin from the Ca^{2+} channels and efficient binding of synaptotagmin in order for fusion to proceed. Thus, sequential Ca^{2+}-dependent interactions of multiple proteins with syntaxin may serve to order the biochemical events leading to membrane fusion.

SPECIFIC INTERACTIONS OF SYNPRINT SITES FROM THE ISOFORMS OF α_{1A} WITH SNARE PROTEINS

Both ω-CTx-GVIA-sensitive N-type and ω-Aga-IVA-sensitive P/Q-type Ca^{2+} channels play a key role in controlling synaptic transmission. However, in contrast to N-type Ca^{2+} channels, the corresponding synprint segment of L_{II-III} from the rbA isoform of α_{1A} [57] does not bind to syntaxin [130]. Examination of the amino acid sequence differences between the BI and rbA isoforms of α_{1A} [57,58] shows considerably lower identity (78%) in L_{II-III} loop than in the remainder of the protein (>98%). The distinctly different levels of amino acid sequence identity in these regions suggest that this loop may be subject to alternative splicing. In support of this idea, we found that α_{1A} isoforms with different amino acid sequences characteristic of L_{II-III} from both rbA and BI are present in both rat and rabbit brain using site-directed antibodies [136]. Thus, the synprint region of the α_{1A} subunit is subject to alternative splicing to yield at least two isoforms.

Fusion proteins containing the intracellular loop L_{II-III} of these two α_{1A} isoforms bind with different affinities to the presynaptic proteins syntaxin and SNAP-25 [132] (Table 2). The BI isoform has higher affinity for both syntaxin and SNAP-25 than the rbA isoform. Under *in vitro* binding conditions, binding of rbA to SNAP-25 is clearly detected, while binding to syntaxin is not [132]. If these binding interactions are required for efficient coupling of Ca^{2+} influx with synaptic vesicle fusion, these data imply that a neuron could modulate the efficiency of synaptic transmission by regulating the expression of different isoforms of a single class A Ca^{2+} channel gene. Consistent with this idea, the BI and rbA isoforms of α_{1A} subunits are differentially distributed at synapses in rat brain [136].

Table 2 Summary of the interactions of Ca^{2+} channel synprint peptides with synaptic proteins

	Syntaxin		*SNAP-25*		*Synaptotagmin*	
	Binding	*Ca^{2+} dep*	*Binding*	*Ca^{2+} dep*	*Binding*	*Ca^{2+} dep*
α_{1B}	−	+	+	+	+	−
$\alpha_{1A(rbA)}$	−	−	+	−	+	+
$\alpha_{1A(BI)}$	+	−	+	−	+	−

Abbreviation: Ca^{2+}dep, Ca^{2+} dependence.

Similar to the synprint site of N-type channels, the synprint site from the BI isoform of α_{1A} involves two adjacent segments of the intracellular loop connecting domains II and III between amino acid residues 722 and 1036 (Fig. 3), and it binds specifically to the C-terminal one-third of syntaxin 1A (amino acid residues 181–288) [132]. These interactions of the BI synprint peptide with both syntaxin and SNAP-25 are competitively blocked by the corresponding synprint region of the N-type channels, indicating that these two channels bind to overlapping or identical regions of syntaxin and SNAP-25 [132]. Collectively, these results provide a molecular basis for a physical coupling of neuronal N-type and P/Q-type Ca^{2+} channels with synaptic vesicle docking/fusion complexes, enabling tight structural and functional association of Ca^{2+} entry sites and neurotransmitter release sites. Differences in the interactions of the synprint sites on α_{1B} and the rbA and BI isoforms of α_{1A} with SNARE proteins may alter the regulatory properties of synaptic transmission at different nerve terminals.

CALCIUM-INDEPENDENT INTERACTIONS OF SNARE PROTEINS WITH THE SYNPRINT PEPTIDES OF P/Q-TYPE Ca^{2+} CHANNELS

Binding of SNARE proteins to the synprint peptides from the rbA and BI isoforms of α_{1A} has different dependence on Ca^{2+} concentration from the synprint peptide of α_{1B} [137]. The BI isoform of α_{1A} binds syntaxin and SNAP-25 in a calcium-independent manner. The rbA isoform of α_{1A} does not bind to syntaxin appreciably in vitro and binds SNAP-25 in a calcium-independent manner. The differences in Ca^{2+} dependence of interaction of these synprint peptides with different SNARE proteins suggests that the Ca^{2+} dependence of synprint interaction is not an essential element of the transmitter release pathway but serves a modulatory role which may confer different regulatory properties on transmitter release mediated by the different presynaptic Ca^{2+} channels.

INTERACTIONS OF THE SYNPRINT SITE WITH SYNAPTOTAGMIN

The vesicle SNARE protein synaptotagmin is thought to serve as a Ca^{2+} sensor for fast neurotransmitter release. Immunochemical studies show that it is associated with purified N-type and P/Q-type Ca^{2+} channels, similar to syntaxin and SNAP-25 [126,128]. The interaction of synaptotagmin with the synprint sites of N-type and P/Q-type Ca^{2+} channels was measured using similar methods as described above [138,139]. Synaptotagmin forms a specific complex with synprint sites from both α_{1A} and α_{1B}. These two synprint peptides compete for binding to synaptotagmin, indicating that they bind to identical or overlapping sites. Moreover, using both immobilized recombinant proteins and native presynaptic membrane proteins, we found that the synprint peptide of N-type channels and synaptotagmin competitively interact with syntaxin [138]. These results predict that, in a nerve terminal, syntaxin molecules bound to Ca^{2+} channels cannot interact effectively with synaptotagmin, an interaction that is thought to be essential for transmitter release.

The competition between the synprint site of α_{1B} and synaptotagmin is Ca^{2+}-dependent because of the Ca^{2+} dependence of the interactions between syntaxin and these two proteins. The affinity of N-type Ca^{2+} channels for binding to syntaxin is modulated by Ca^{2+} concentration, with maximal binding at a range of 10–30 μM near the threshold for neurotransmitter release [131]. In contrast, maximum binding of syntaxin to synaptotagmin I and II requires higher concentrations of Ca^{2+} in the range from 100 μM to 1 μM [105–107,140]. As the Ca^{2+} concentration increases beyond 30 gmM, interaction of syntaxin with the synprint site of N-type Ca^{2+} channels will be weakened and interaction with synaptotagmin will be strengthened. Thus, these studies provide potential biochemical correlates for the sequence of events during synaptic vesicle exocytosis: binding of syntaxin and SNAP-25 to N-type Ca^{2+} channels at low Ca^{2+} concentration, enhanced affinity of that interaction at Ca^{2+} concentrations in the range of 10–30 μM, and displacement of the synprint binding interaction on syntaxin by synaptotagmin at Ca^{2+} concentrations in the range of 100 μM and higher. This sequence of protein–protein interactions with N-type Ca^{2+} channels may serve to control the triggering of exocytosis by regulating the interaction of syntaxin with synaptotagmin (Fig. 4).

Although binding of synaptotagmin to the α_{1B} subunit of N-type Ca^{2+} channels is calcium-independent, its binding to the rbA isoform of the α_{1A} subunit of P/Q-type Ca^{2+} channels is calcium-dependent with maximum binding at 10–30 μM calcium, similar to the Ca^{2+} dependence of binding of syntaxin and SNAP-25 to α_{1B} [137]. In contrast, synaptotagmin binding to the BI isoform of α_{1A} is calcium-independent. How might the difference in Ca^{2+} dependence of interactions with the SNARE proteins affect transmitter release initiated by N-type and P/Q-type Ca^{2+} channels? Because fast transmitter release is triggered by Ca^{2+} concentrations in the range of 100 μM or more, it is unlikely that the differences in the Ca^{2+} dependence of interaction of synprint sites with SNARE proteins at 10–30 μM Ca^{2+} is a key element in the basic transmitter release process. Instead, we propose that these calcium-dependent interactions are important for calcium-dependent modulation of transmitter release by post-tetanic potentiation and related short-term processes which modulate transmitter release in response to

sustained changes in Ca^{2+} concentration in the 1–30 μM range [141]. Our results predict differences in the Ca^{2+} dependence and molecular mechanisms of these calcium-dependent forms of synaptic plasticity based on the differences in Ca^{2+} dependence of interaction of the synprint sites of Ca^{2+} channel subtypes with SNARE proteins.

PHYSIOLOGICAL SIGNIFICANCE OF THE INTERACTION OF N-TYPE Ca^{2+} CHANNELS WITH SNARE PROTEINS IN SYNAPTIC TRANSMISSION

The structural and functional coupling between Ca^{2+} entry sites and release sites of docked synaptic vesicles would ensure that neurotransmitter release is triggered rapidly when the action potential invades the nerve terminal. Although biochemical data support the hypothesis that there is a tight association between Ca^{2+} channels and exocytotic apparatus, the functional roles of synaptic protein-Ca^{2+} channel interactions in calcium-triggered exocytosis remain to be determined. Our biochemical results predict that peptides containing synprint sites, if injected into neurons, would inhibit synaptic transmission by competitively binding to syntaxin and SNAP-25. This would prevent SNARE protein binding to presynaptic Ca^{2+} channels, increasing the distance between docked vesicles and Ca^{2+} channels and increasing the requirement for Ca^{2+} influx to initiate transmitter release (Fig. 5). To test this hypothesis, we injected competing peptides into the presynaptic cells of both sympathetic ganglion neuron synapses and *Xenopus* embryonic neuromuscular junctions in culture and examined the functional consequences [142,143].

Cultures of superior cervical ganglion neurons (SCGNs) are favorable for functional tests of peptide blockers of transmitter release. Peptides can be introduced into the relatively large (30–40 μM) presynaptic cell bodies by microinjection, the injected peptides can rapidly diffuse down short axons to nerve terminals forming synapses with adjacent neurons, the effects on stimulated release of acetylcholine can be accurately monitored by recording the excitatory postsynaptic potentials (EPSPs) evoked by action potentials in presynaptic neurons, and only N-type Ca^{2+} channels control Ach release at these synapses so a homogeneous population of channels can be studied. Synaptic transmission was monitored between closely spaced pairs of neurons for 20–30 min, and then peptides containing synprint site were allowed to diffuse into the presynaptic neurons from a suction pipette for 2–3 min. EPSPs were evoked by action potentials elicited by current pulses applied to the presynaptic cell through a recording microelectrode and were recorded with a second microelectrode in the nearby postsynaptic cell [144,145]. Peptides containing the synprint site from the α_{1B} subunit disrupt the interaction of native N-type channels with syntaxin and reduce synaptic transmission in SCGN synapses by up to 50% for different synprint peptides, without any effect on Ca^{2+} currents [142]. Rapid, synchronous synaptic transmission is inhibited, while late, asynchronous EPSPs and paired-pulse facilitation are increased, consistent with the conclusion that synaptic vesicles are shifted from a pool primed for synchronous release to a pool that is not optimally primed or positioned

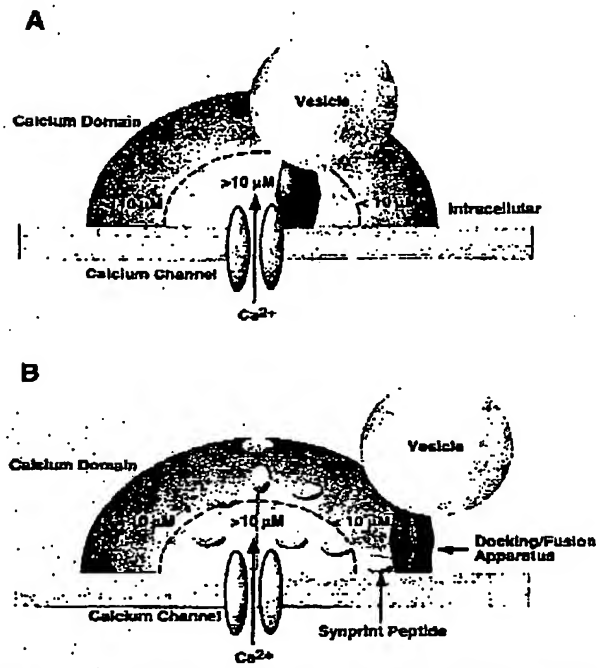

Fig. 5 The proposed inhibitory role of injected peptides containing N-type synprint site on neurotransmission of SCG neurons and neuromuscular junctions. (A) The physical link between N-type Ca^{2+} channels and synaptic vesicle docking/fusion apparatus. Exocytosis of synaptic vesicles requires high Ca^{2+} concentration, with a threshold of 20–50 μM and half-maximal activation at 190 μM. The brief rise in Ca^{2+} concentration to the level necessary for exocytosis likely occurs only in proximity to the Ca^{2+} channels, since the intracellular Ca^{2+} concentration falls off steeply as a function of distance from the source of Ca^{2+}. Thus, when binding to the synprint site, synaptic vesicles are docked in proximity to Ca^{2+} entry sites. Upon Ca^{2+} influx, the fusion apparatus is activated for rapid, Ca^{2+}-dependent, synchronous synaptic transmission. (B) The peptides containing the synprint site competitively block the physical link between N-type Ca^{2+} channels and synaptic vesicle docking/fusion apparatus, and subsequently remove pre-docked vesicles away from Ca^{2+} entry sites. This decreases the degree of efficiency by shifting Ca^{2+}-dependence to higher values. Rapid, synchronous synaptic transmission is inhibited, while late, asynchronous EPSPs and paired-pulse facilitation are increased, consistent with the conclusion that synaptic vesicles are shifted from a pool primed for synchronous release to a pool not optimally primed or positioned for synchronous release.

for synchronous release (Fig 5). The corresponding peptides from L-type Ca^{2+} channels have no effect on EPSPs. The relative efficiency for inhibition of transmitter release by three different peptides, L_{II-III} (718–963) > L_{II-III} (832–963) > $L_{II\ (718-859)}$ is consistent with their rank order of affinity for *in vitro* binding with syntaxin [142]. These results provide direct evidence that binding of presynaptic Ca^{2+} channels to the synaptic docking/fusion complex is required for rapid, synchronous neurotransmitter release.

Early work at the frog neuromuscular junction revealed that fast synaptic transmission is steeply dependent on the external Ca^{2+} concentration $[Ca^{2+}]_e$ and the probability of acetylcholine release at the frog neuromuscular junction increases as the fourth power of $[Ca^{2+}]_e$ [146]. To determine whether the inhibition of synaptic transmission by synprint peptides might be due to displacement of the docked vesicle away from the Ca^{2+} channels and resultant changes in the Ca^{2+} dependence of transmission, we used *Xenopus* nerve-muscle cocultures from developing embryos in which synaptic transmission is mainly dependent on N-type Ca^{2+} channels [147]. This is an ideal preparation to study of the effects of N-type synprint peptides on synaptic transmission because the Ca^{2+} transients in the presynaptic terminal can be imaged in parallel with measurements of synaptic transmission. During the first days of development, the embryos undergo cell divisions without substantial growth. Injection of synprint peptides into early blastomeres leads to loading of all progeny cells, including spinal cord neurons and muscle cells, without much dilution by cell expansion. Following cell culture, synaptic transmission of peptide-loaded and control cells were compared by measuring postsynaptic responses under different external Ca^{2+} concentrations. The dependence of synaptic transmission on Ca^{2+} concentration was shifted to higher concentrations so that, at physiological Ca^{2+} concentrations, approximately 50% reduction of transmitter release of injected neurons was observed. Analysis by a theoretical model indicated that at least 70% of the docked vesicles were detached from Ca^{2+} channels under these conditions. High Ca^{2+} concentrations can overcome this inhibition. Injection of the corresponding region of the L-type Ca^{2+} channels had virtually no effect [143]. These data suggest that disruption of the physical link between N-type Ca^{2+} channels and synaptic vesicle docking/fusion apparatus displaces pre-docked vesicles from Ca^{2+} entry sites, making neurotransmitter release less efficient by shifting its Ca^{2+}-dependence to higher values (Fig. 5). These findings are consistent with the functional data on rat superior cervical ganglion neurons [142] where a maximum of 42% inhibition of synaptic transmission was observed following diffusion of synprint peptides.

Ca^{2+} CHANNEL MODULATION BY INTERACTION WITH SNARE PROTEINS

In addition to the functional role of interaction between Ca^{2+} channels and SNARE proteins in the anterograde process of synaptic transmission, these interactions also have retrograde effects on Ca^{2+} channel function. In *Xenopus* oocytes, co-expression of syntaxin with N-type or P/Q-type Ca^{2+} channels reduces the level of channel expression and inhibits Ca^{2+} channel activity by shifting the voltage dependence of steady-state inactivation during long depolarizing prepulses toward more negative membrane potentials [148,149]. These inhibitory effects are relieved by co-expression of synaptotagmin [150], presumably by competitive inhibition of syntaxin binding to Ca^{2+} channels as observed in biochemical experiments [138]. In contrast to the effect observed in *Xenopus* oocytes, proteolytic cleavage of syntaxin in isolated calyx nerve terminals by treatment with botulinum toxin prevents inhibition of Ca^{2+} channels by G proteins [151]. Thus, in this more physiological preparation, syntaxin does not alter basal Ca^{2+} channel function, but it is required for normal regulation by activation of G proteins.

Ca^{2+} CHANNEL INTERACTIONS WITH SNARE PROTEINS IN TRANSMITTER RELEASE

Several lines of evidence suggest that Ca^{2+} binding to synaptotagmin is part of the signal that initiates rapid exocytosis [104,108,110,133,134]. Our studies show that the interaction of N-type Ca^{2+} channels with the synaptic core complex is also dependent on changes in Ca^{2+} concentration near the threshold level for initiation of transmitter release and that binding of the Ca^{2+} channel to syntaxin prevents interaction of syntaxin with synaptotagmin. We propose that docked vesicles form a low-affinity complex with the N-type Ca^{2+} channels through binding to syntaxin and SNAP-25 at resting Ca^{2+} concentration. Ca^{2+} influx increases the affinity of this coupling, so the energy of Ca^{2+} binding to this complex may contribute to the energetic driving force for the early steps of the fusion process. Finally, as the free Ca^{2+} reaches the threshold for release (20–50 μM), the binding affinity of the synprint site is reduced, syntaxin and SNAP-25 dissociate from the Ca^{2+} channel, and synaptotagmin binds to the complex of syntaxin and SNAP-25. Higher levels of Ca^{2+} (above 30 μM) may be needed to enable displacement of syntaxin from the N-type Ca^{2+} channels and allow efficient binding of synaptotagmin in order for fusion to proceed. Thus, sequential Ca^{2+}-dependent interactions of multiple proteins with syntaxin may serve to order the biochemical events leading to membrane fusion (Fig. 4).

For presynaptic Ca^{2+} channels containing α_{1A} as their pore-forming subunit, different calcium-dependent interactions are observed *in vitro*. We propose that the sequence of interactions of syntaxin first with the synprint site of presynaptic Ca^{2+} channels and then with synaptotagmin remains the same and is an essential element of the release pathway. For the BI isoform of α_{1A}, no calcium-dependent interactions are observed *in vitro*, so it is likely that synaptotagmin displaces the synprint site from syntaxin without the aid of a calcium-dependent decrease in binding affinity between the synprint site and syntaxin. This may require a higher concentration of Ca^{2+} to stimulate the interaction of synaptotagmin and syntaxin and therefore increase the steepness and cooperativity of the Ca^{2+} dependence of neurotransmitter release mediated by the BI isoform of α_{1A}. For the rbA isoform of α_{1A}, a biphasic dependence of binding to synaptotagmin is observed, but there is no calcium-dependent binding to SNAP-25 or syntaxin. In this case, calcium-dependent release of synaptotagmin from interaction with the synprint peptide may allow the interaction of synaptotagmin with syntaxin to occur more easily as Ca^{2+} concentrations increase and thereby enhance the release process. The differences in calcium-dependent interactions of these synprint sites with SNARE proteins may allow differential modulation of the release process by Ca^{2+} and perhaps by other influences such as protein phosphorylation and G proteins.

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